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(71) Applicant (*for all designated States except US*): BIOINVENT INTERNATIONAL AB [SE/SE]; Sölvegatan 41, S-223 70 Lund (SE).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): BORREBAECK, Carl, A., K. [SE/SE]; Åttevägen 8 A, S-245 62 Hjärup (SE).

(74) Agent: MEWBURN ELLIS; York House, 23 Kingsway, London WC2B 6HP (GB).

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(54) Title: IMPROVED METHOD OF SELECTING SPECIFIC BACTERIOPHAGES

(57) Abstract

An improved method for selecting a molecule such as an antibody, antigen, peptide, protein or fragment thereof and its encoding sequence, which molecule is expressed together with a phage coat protein on the phages surface. The improvement is achieved through a new mutant filamentous helper phage which has retained the gene III promoter, whereas the gene III encoding sequence is deleted. Amplification of phage titer of 10^6 times were achieved in M13-derived phages, when used for the selection of specific antibody.

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IMPROVED METHOD OF SELECTING SPECIFIC BACTERIOPHAGES

Technical area of invention

This invention relates to the selection of specific binding proteins and their genes using a new mutant bacteriophage in phage display techniques.

Background of the invention

In our patent application PCT/SE94/01166 we described a method of selection of antigenic or other specific binding protein molecules, and their encoding DNA, by means of a phage display package produced by co-expression of a phagemid vector and a helper phage, in which the DNA encoding the binding peptide is inserted into the phagemid vector so as to be expressed as a fusion with a truncated phage gene III protein. The resulting display package lacks functional protein III, and is therefore not infectious, but it can be selectively rendered infectious by means of a fusion protein comprising functional protein III and the complementary binding partner for the specific binding protein present on the surface of the phage display package. The function of the helper phage is to provide the genes necessary for packaging the expression products of the phagemid; but it will be deficient in gene III so that the resulting phage display package will not contain functional gene III.

A similar system has been disclosed also in example 2 of EP-A-614989. In that case, the helper phage used was a derivative of phage Fd tet, known as fKN16, which had a deletion of 507 nucleotides in gene III. In our PCT application we used a derivative of phage M13 KO7 having a 1121 nucleotide deletion in gene III between nucleotides 1525 and 2646.

We have found that this M13 helper phage is considerably better than phage fKN16 in the performance of the procedure. Not only that, we have now also found that it is similarly better than another Fd phage (fCA55) having a larger deletion in gene III; and furthermore, that we can improve on our M13 phage construction previously described.

The experimental basis for this is presented below. In summary, however, the following general features seem to emerge.

Definition of the invention

Firstly, whereas in our original delta 3 version of the M13 helper phage, we had deleted the gene III promoter and most of the coding sequence, in our new delta 3.2 version we kept the gene III promoter but deleted effectively all the gene III coding sequence. Both constructs had the effect of preventing expression of any gene III protein, but the delta 3.2 version, by keeping the gene III promoter, retained the small intergenic (IG) region between gene VIII and gene III. It appears that this is at least in part responsible for the improved performance of the delta 3.2 helper phage over the delta 3 phage. Accordingly, the design of a helper phage for use in this type of selection procedure should take account of this, and substantially retain the small IG region between gene VIII and gene III.

It may not be necessary to retain the entire small IG region. This region contains a number of functional motifs. For example, as well as the promoter for gene III, there is at least one transcription termination sequence. Precisely what aspect of the small IG region is important remains to be seen, but the minimum sequence required from this region could if desired be determined by routine experimentation.

Secondly, while this development has been discovered in connection with the M13 phage, it seems likely that a similar phenomenon will be observed with other filamentous helper phages. Examples of such are fd, f1, 1f1, 1ke, ZJ/2, Xf, Ff, Pf1 and Pf3. Thus, optimal function of for example Fd-type phages in this procedure might require retention of the small IG region (along with other features).

Thirdly, insofar as retention of the small IG region involves retention also of the promoter for gene III, it is probably important that there be no gene III coding sequence, or even perhaps other coding sequence, expressible from the transcript from that promoter. This would suggest deletion of all gene III coding sequence and/or ensuring that there is no translational

start codon in the transcript such as would give a protein or peptide which could interfere with the function of the helper phage and the proper packaging of the display phage.

Fourthly, as shown in figure 3, the M13 KO7 phage contains the kanamycin resistance gene and the p15A origin of replication inserted in the large IG region of the phage. It is believed that this too could play a significant part in the advantage which the M13 phage has over the Fd phage in the procedure of the present invention. In that case, Fd-type or other phages could be modified to incorporate an insert into the large IG region which is similar to part or all of that present in M13 KO7.

Detailed description of the invention

The main aim of the present invention is to make available a highly efficient helper phage . It has been found that a surprisingly much more efficient selection of the phages expressing a specific binding protein or ligand I on their surface can be achieved by using a bacteriophage mutant, wherein the gene III promoter (included a small intergenic region between gene VIII and gene III) is retained, whereas gene III is deleted.

Another aspect of the invention is the use of the new mutant in a method for the improved selection of a specific binding protein or ligand I, which is expressed together with a phage coat protein on the surface of a phage characterised by , linking of specific phage replication and recognition of ligand I on the phage surface by:

a) letting a helper phage stock, which phages have kept the gene III promoter, whereas the gene III encoding sequence is deleted, but carry protein 3 on their coats, infect bacteria which comprises a phagemid vector with cloned ligand I

b) adding a fusion protein between protein 3 or a part thereof and a ligand II specifically interacting with said ligand I, so that ligand I and ligand II bind specifically to each other and thereby also adding protein 3 to those specific phages which carry ligand I

c) letting said phages which carry ligand I, ligand II and protein 3 on their surface infect bacteria and thereby replicate and multiply.

A further aspect of the invention is a kit comprising the new mutant.

The expression "specific binding protein" is covered by the expression "ligand I" which is represented by a peptide, a protein, an antibody or an antigen or another specific binding molecule. Ligand I is preferably an antibody and more preferably a human antibody.

"Ligand II" is a molecule such as a peptide, protein, organic molecule, hormone or other molecule specifically interacting with ligand I and capable of being linked to protein 3 in the fusion protein. Ligand I and II are thus referring to receptor-ligand pair of molecules such as antibody-antigen, hormone-receptor-hormone, growth factor-receptor-growth factor, substrate-enzyme or avidine-biotin.

The above mentioned expressions do cover functional parts thereof meaning fragments retaining the binding ability of the protein, ligand I or ligand II to about the same extent.

Description of the figures

Figure 1. PCR amplification of the deleted phages from residue 1301 to 2871, which included gene VIII and III, with the intergenic region. Lane 1: M13 KO7; Lane 2: fKN 16; Lane 3: fCA 55; Lane 4: M13MDΔ3; Lane 5: M13MDΔ3.2; Lane 6: Negative Control, Lane 7: Molecular mass markers (bp).

Figure 2. Selection and amplification of phages (SAP) using the four helper phage constructs for phagemid particle preparation. The values represent the mean of three different experiments. (●) fKN 16, (Δ) fCA 55, (■) M13 MDΔ3) and (▲) M13 MDΔ3.2.

Figure 3. The genetic map of the vector M13KO7 (Vieira and Messing 1987).

Examples

Phagemid vectors for display of proteins/peptides on the surface of filamentous phage utilize a plasmid genome carrying the phage origin of replication, along with the gene fused to a fragment of gene III. Generation of phage particles displaying the fusion protein also requires superinfection of the host bacterium with a helper virus. We describe here the construction of a new gene III mutant of M13 KO7 bacteriophage and compare its ability to act as helper phage with two mutants derived from Fd tet (fKN 16 and fCA 55). Furthermore, we investigate their capability to act as helper phages in SAP-selection, where non-infectious helpher phage, expressing antibody fragments but not protein 3, still can infect by first reacting with a soluble antigen-protein3 fusion protein. Gene III mutants were found to be non-infectious and high titer of infective particles were obtained only when the helper phage was grown in cells harbouring a gene III-containing plasmid. Amplification of phage titer of 10^6 times were achieved in M13-derived phages, when used for the selection of specific antibody fragments.

Introduction

Phage display of antibody fragments (1), peptides (2) and hormones (3) has been demonstrated to be a very useful technology for the rescue of specific binders from large combinatorial libraries. Two of the bacteriophage coat proteins, the major protein encoded by gene VIII (p8) and the minor protein encoded by gene III (p3), have surface-exposed N-terminal domains that tolerate foreign protein inserts and have been used to display fusion proteins. For the display of antibody fragments the p3 molecule has predominantly been used as fusion partner since only three or five copies are packed per virion. During the past five years, many improvements of the original display approach have been reported. In particular, the use of small phagemid vectors that allows packing of a mixture of wild type p3 and fusion protein has faciliated the approach.

We have recently developed a new system for selection and amplification of phages (SAP) for antibody libraries (4), in which p3-deleted, non infectious phage particles become infective by a specific interaction between the displayed antibody fragment and a soluble protein consisting

of the antigen fused to the N-terminal part of p3. For this purpose we generated a mutant bacteriophage (M13MDΔ3) with a deletion in gene III and in the intergenic region (IG) between gene VIII and gene III (5). After superinfection this helper phage provided all proteins and packing signals except the wildtype p3. In the present study, we constructed and evaluated different deleted helper phages from M13 and compared these with the related Fd tet phages. We demonstrate the importance of the small IG region for correct packing and study how interference resistance of the helper phage affects the selection of phagemid particles expressing antibody fragments.

Example 1

Vector constructions, Materials and methods

Bacterial and phage strains: *Escherichia coli* K-12 strain XL1-Blue (6) F′ :: Tn10 *pro*A^+B^+ *lac*I^q Δ(*lac*Z) M15/*rec*A1 *end*A1 *gyr*A96 (Nal^r) *thi hsd*R17 ($r_k^- m_k^+$) *sup* E44 relA1 *lac* and TG1 (7) F′ traD36 *lac*I^q Δ(lacZ)M15 pro A^+B^+/*sup*E Δ (*hsd*M-*mcr*B)5($r_k^- m_k^- McrB^-$) thi Δ (*lac*-proAB)were used as indicator bacteria and/or producer of bacteriophages. Bacteriophages derivated from Fd tet, the fCA 55 and fKN 16, have been described by Crissman and Smith (8), the M13 KO7 phage by Vieira and Messing (9) and the M13 MDΔ3 by Dueñas and Borrebaeck (4).

Construction of M13-derived gene III mutant: The deleted phage, denoted M13MDΔ3.2 was constructed by a complete PCR amplification of the genome of the M13 KO7 bacteriophage, excluding only gene III, thus, keeping the IG region between genes VIII and III intact. The primers used for the PCT amplification were the 5′ (BamHI)
CGGGATCCATGCCAGTTCTTTTG GGTA the 3′ (BamHI)
CGGGATCCGTTGAAAATCTCCAAAAAAAAA GGCT the reaction was done in 30 cycles of 1 min. 94°C, 1 min. 55°C and 9 min. 72°C. The PCR product was digested BamHI over night at 37°C and ligated and transformed into TG1 cells containing a pUC-gIII plasmid (kindly provided by Dr. H. Hoogenboom.)

Results

Construction of M13 derived bacteriophages with a deleted gene III.

We have constructed two truncated (gene3 deleted) versions of M13 KO7 constructed and assembled by PCR. The M13MDΔ3 phage, containing a deletion of 1121 bp (from 1525 to 2646), can be used as a helper phage for superinfection if initially produced by a cell transcribing the gene III product on a separate plasmid. However, the amount of infectious particles formed was very low and only 10^5 cfu/ml could be obtained. In order to evaluate if the small IG region between genes VIII and III was responsible for the low phage titer, we constructed a second version of the deleted M13 denoted M13MDΔ3.2. This second mutant M13 lacked only gene III, i.e.. from residue 1579 to 2851. When M13MDΔ3.2 was used as helper phage titers as high as 10^9 cfu/ml were obtained.

Effect of gene III deletion on infectivity of M13 and Fd filamentous bacteriophages. M13MDΔ3, M13MDΔ3.2 and two derivatives of Fd tet bacteriophages (fKN 16 and fCA 55, which carried deletions of 507 and 930 bp in gene III, respectively) were used in order to examine their ability to infect E. coli expressing F′pili. All deletions were verified by PCR of the relevant phenotypes (Figure 1).

The ability of both the gene III deleted M13 and Fd tet phages to infect F'^{+} E. coli (TG1 or XL1-Blue) strains grown in tetracycline, was abolished. However, when these phages were produced in bacteria harbouring a pUC-gIII plasmid, which provided p3 in a transcomplementation to the phage particle, the non-infectious phenotype was overcome by the presence of p3 in the viral coat. However, only the deleted genome was incorporated into the phage, since the pUC-gIII plasmid does not carry the filamentous phage origin of replication and packing signals. The progeny phages were thus able to infect only once since non-infectious particles were subsequently assembled. Table I shows the yield of infectious particles obtained with or without the co-expression of p3. Even though the infectivity of all mutant phages was restored by incorporating p3, not all of them were efficiently assembled. The M13MDΔ3 phage formed only 10^5 cfu/ml in contrast to M13MDΔ3.2, which was assembled similarily to the fCA 55 phage.

Example 2

Helper Phage, fusion protein and SAP-selection

Helper phage and noninfective phage stocks were prepared as described (4), using the four mutant helper phages derived from M13 and Fd, tet. of Example 1. Briefly, E. coli containing a plasmid carrying gene III were transformed with the differently deleted bacteriophages. After over night incubation the supernatant containing the helper phages was titrated using XL1-Blue as indicator bacteria. Cells harbouring a phagemid encoding for anti-hen egg lysozyme (HEL) or anti-phenyloxazolone Fab fragments, fused with the carboxy-terminal part of p3, were infected with the mutant helper phages, thus, creating non-infectious phage stocks. A gene coding for HEL and a 98 amino acid fragment of M13 p3 was assembled by PCR, using the primers described in (4) and cloned into a pUC19-based plasmid. The fusion protein was expressed as a soluble periplasmic protein. The characterization of the fusion protein as a bridging molecule and the SAP procedure was performed, as previously described. (4). The specific amplification was determined as the increase in titer of the anti-HEL phage preparation over the non-specific anti-phenyloxazolone, using different amounts of fusion protein. The amount of fusion protein is given in % (v/v).

Example 3

Comparison of mutant phages in SAP selection.

Phagemid stocks were prepared displaying antibody Fab fragments specific for HEL and phenyloxazolone. These were specifically amplified and selected using a HEL-p3 fusion protein as a bridging molecule between the F'pili and the non-infectious phagemid particle (4). Different dilutions of the fusion protein were used and the phage titers are shown in Figure 2. M13MDΔ3 and M13MDΔ3.2 yielded a specific phage amplification of 5×10^5 and 2×10^6-fold, respectively, whereas the Fd-derived phages only gave 100-fold amplification over a high background of non specific infections. High concentrations of the fusion protein (above 30%) caused an inhibitory effect most probably due to a depolymerization of the bacterial pili by the p3 fragment, thus, limiting the infection process.

Discussion

Previous studies have shown that non-polar mutants of gene III of M13 (10) or Fd tet (8) produced defective phage particles and accordingly ascribed two functions to the gene III product: infectivity and normal (non-polyphage) morphogenesis. The finding that the fKN 16 phage could not infect via F′pili, but still retained its ability to form normal particles, suggested that the intact carboxy-terminal domain allows it to carry out the morphogenic function of p3 (8). We constructed and evaluated two non-polar mutants of M13 KO7, where the coexpression with recombinant p3 allowed generation of "normal" particles. The first construction, carrying a deletion comprising the small intergenic region between gene VIII and gene III, did not pack efficiently which resulted in a 10^4-fold reduction in phage titer, as compared to wildtype M13 KO7. This non-coding region contains the central terminator of transcription (5). This was shown by analysis of in vitro transcripts coupled to transcription-translation, where initiation occur at nine different sites along the viral genome but, in absence of *rho*, all in vitro transcripts terminated at this unique terminator site (11). Also, this region contains the promoter preceding gene III, that can generate differences in the amount of gene VI, I and IV transcripts. Consequently the M13MDΔ3.2 helper phage was constructed where the IG region was kept intact. The dramatic increase in titers of infectious particles might be due to the generation of normal levels of mRNA coding for protein I, IV or VI, which have been shown to be important for the correct assembly of phage particle. Since it has been reported that gene III mutants of the Fd tet phage were more likely to tolerate aberrations in gene III without killing its host than wild type M13 (12), we compared our constructions with the fd-derived mutants fKN and fCA 55. The data showed that we indeed could obtain similar titers of Fd tet as compared to non-deleted M13 helper phage.

However, to be useful as a helper phage when constructing antibody libraries, non-interference of the assembly and capability to be rescued after antigenic selection was needed. It was obvious that only the M13 KO7 derivatives that had been engineered to interference resistance by introducing a selection marker and origin of replication between the two domains of the IG region and protein 2 overexpression (9) were able to produce a high yield of phages when they were selected and amplified with the fusion protein. Thus, efficient assembly of the

foreign DNA (i.e. the phagemid vector) into phage particles depended on its preferential packing over the phage genome.

In summary, we can conclude that M13-derived mutant bacteriophages could be efficiently used as helper phages in a method for the selection and amplification of a specific binding protein and preferably in the SAP selection and that the IG region between gene VIII and gene III was needed for an efficient assembly of the phage particle.

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Table 1. Effect of co-expression of protein 3 on the helper phage titer (cfu/ml).

PHAGE	TG1	TG1+GIII	XL1 Blue	XL1 Blue +GIII
M13 KO7	3×10^{11}	5×10^{9}	1×10^{11}	8×10^{8}
fKN 16	3×10^{4}	2×10^{10}	ND	ND
fCA 55	$<10^{2}$	5×10^{8}	ND	ND
M13 MDΔ3	$<10^{2}$	4×10^{5}	$< 10^{2}$	2×10^{5}
M13 MDΔ3.2	$<10^{2}$	3×10^{8}	$<10^{2}$	2×10^{9}

ND = No determined.

Claims

1. Bacteriophage with improved efficiency as a helper phage in the selection and amplification of a gene encoding a specific binding protein, or a functional part thereof, characterised in that
the gene III promoter is kept, whereas the gene III encoding sequence is deleted.

2. Bacteriophage as claimed in claim 1, wherein
the phage is a mutant of a filamentous bacteriophages such as M13, fd, fl, 1fl,1ke, ZJ/2, Xf, Ff, Pfl and Pf3.

3. Bacteriophage according to claim 1 or 2, wherein
the phage is a mutant of the bacteriophage M13 in which a small intergenic region, or a functional fragment thereof ,between gene VIII and gene III is retained.

4. Bacteriophage according to any preceding claim, wherein
the bacteriophage is the mutant M13MDΔ3.2 of the bacteriophage M13 KO7.

5. A method for the improved selection of a specific binding protein or ligand I, which is expressed together with a phage coat protein on the surface of a phage characterised by , linking of specific phage replication and recognition of ligand I on the phage surface by :

a) letting a helper phage stock, which phages have kept the gene III promoter, whereas the gene III encoding sequence is deleted, but carry protein 3 on their coats, infect bacteria which comprises a phagemid vector with cloned ligand I

b) adding a fusion protein between protein 3 or a part thereof and a ligand II specifically interacting with said ligand I, so that ligand I and ligand II bind specifically to each other and thereby also adding protein 3 to those specific phages which carry ligand I

c) letting said phages which carry ligand I, ligand II and protein 3 on their surface infect bacteria and thereby replicate and multiply.

6. A method according to claim 5, wherein
the helper phage is a bacteriophage according to the claims 1-4.

7. A method according to claim 5 characterised in that
ligand I is selected from the group of peptide, protein, antibody, antigen or fragments thereof.

8. A method according to claim 5, characterised in that
ligand II is selected from the group of peptide, protein or fragment thereof, organic molecule, hormone or other molecule which interacts specifically with ligand I and is linked to protein 3 in the fusion protein.

9. A method according to claim 5 and 7, characterised in
using a human antibody or functional fragment thereof as ligand I on the phage surface.

10. A kit for the improved selection and amplification of genes encoding a specific binding protein or ligand I comprising :
at least
a) a helper phage stock , which phages have kept the gene III promoter whereas the gene III encoding sequence is deleted, but which carry protein 3 on their coats enabling them to infect a bacteria host once
and optionally
b) a bacteria which comprises a phagemid vector with cloned ligand I
and/or
c) a fusion protein between protein 3 or a part thereof and a ligand II specifically interacting with said ligand I expressed on the bacteria surface
and/or
d) a bacteria capable of being infected by the phages which carry ligand I, ligand II and protein 3 on their surface and thereby replicate and multiply in said bacteria.

11. A kit according to claim 10, wherein
said helper phage is selected from the bacteriophages of the claims 1 - 4.

12. A kit, according to claim 11, wherein said helper phage is the mutant M13MDΔ3.2.

Figure 1

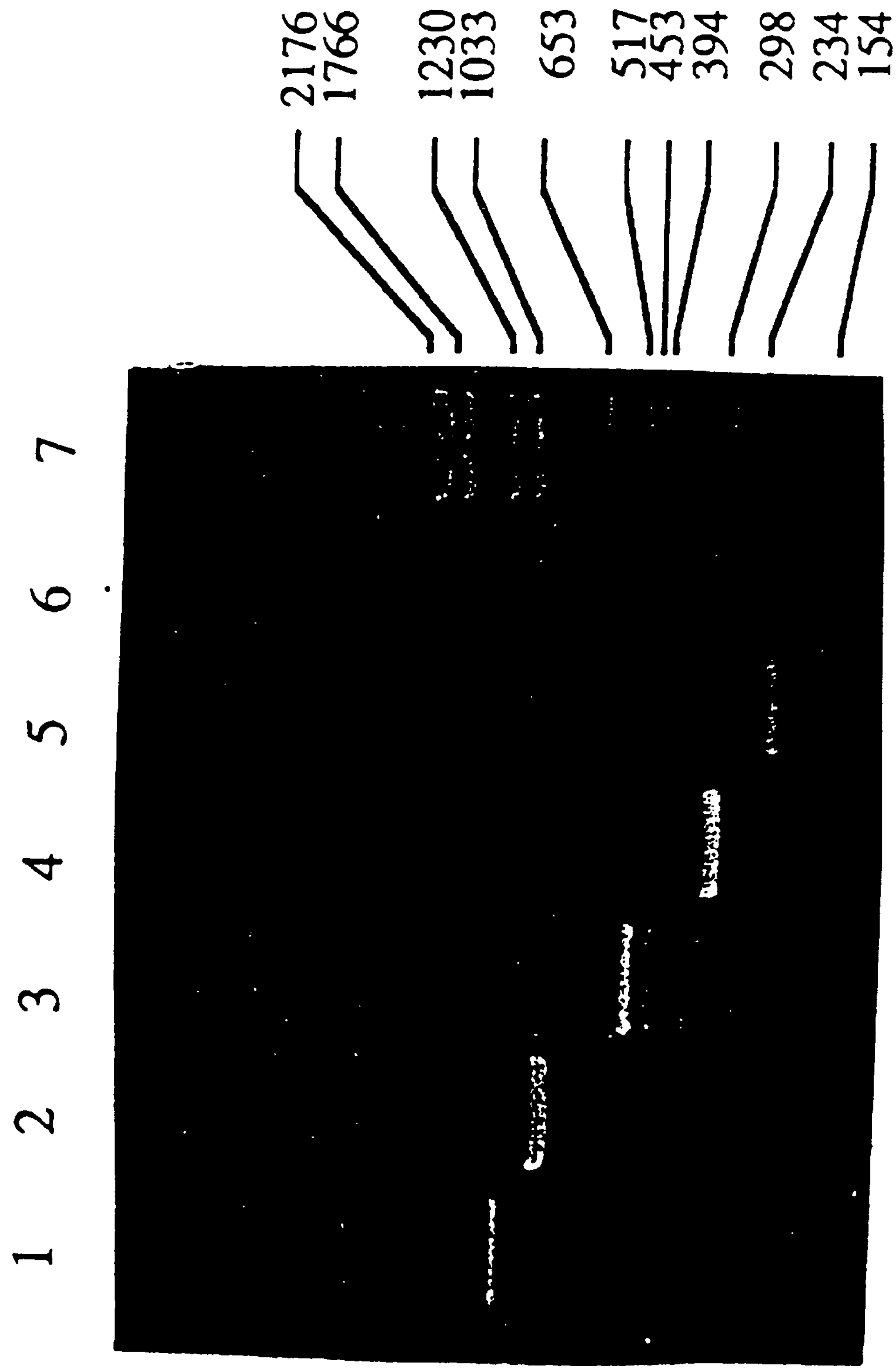


Figure 2

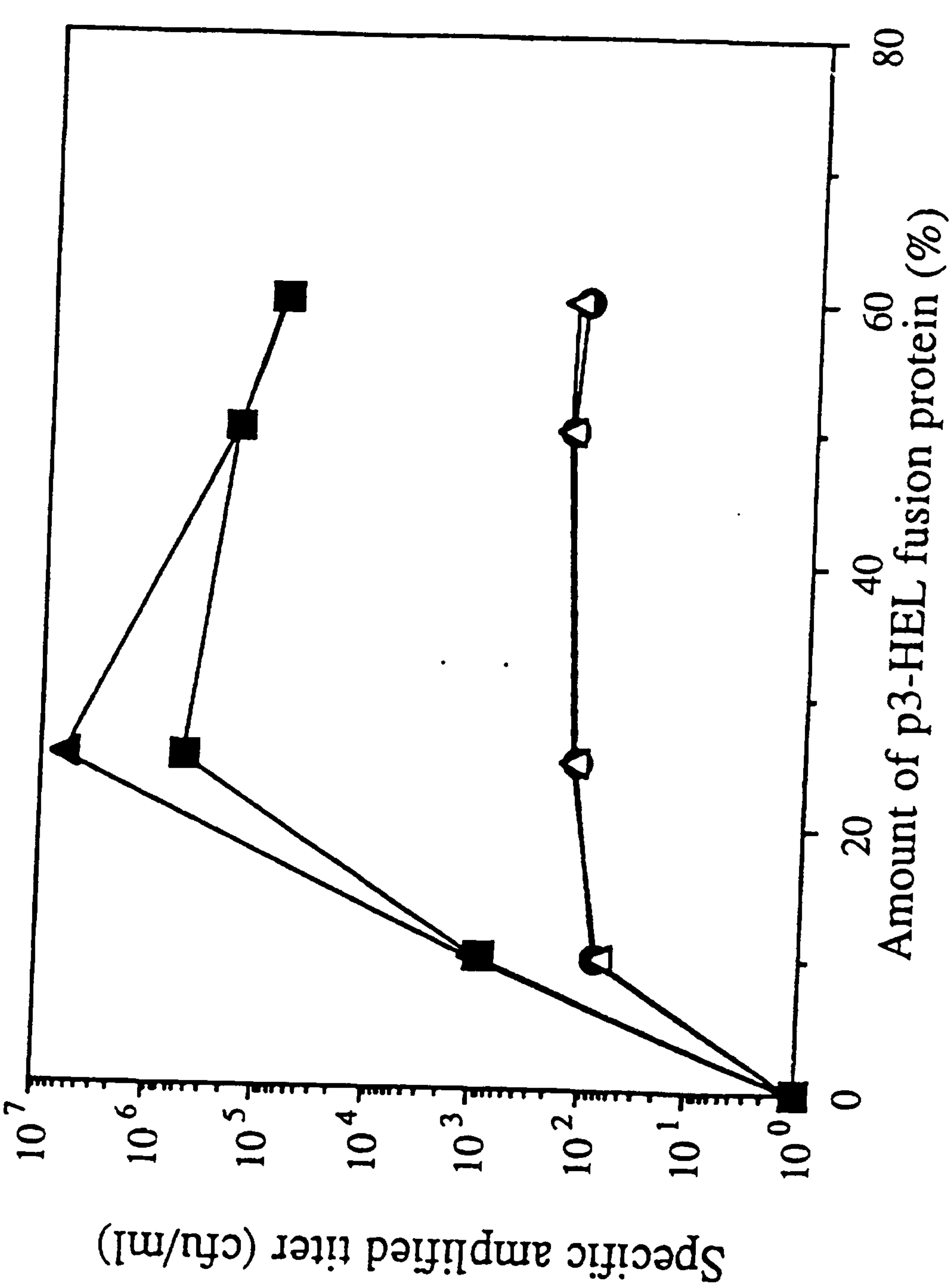
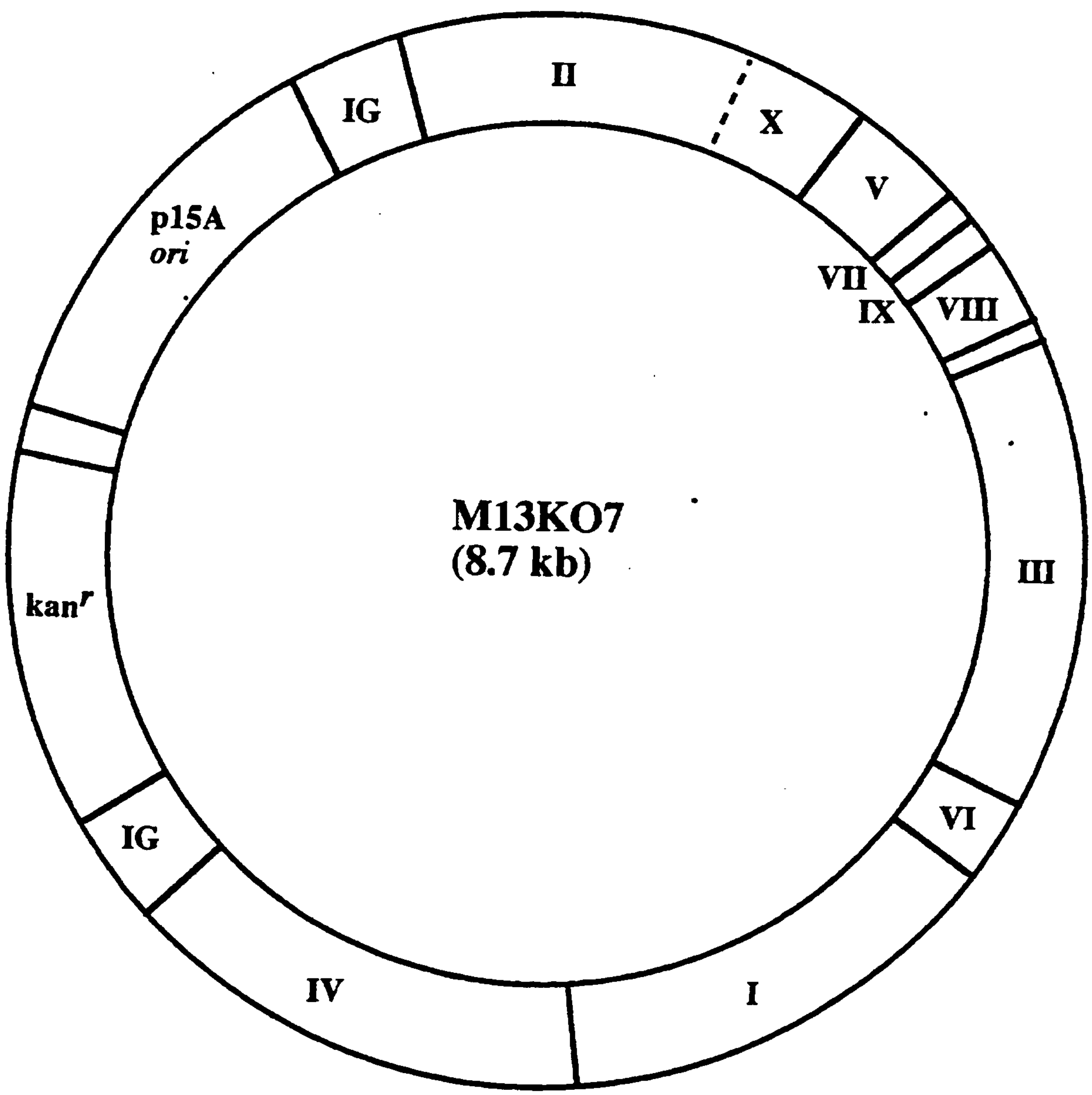

Specific amplified titer (cfu/ml)
10^0
10^1
10^2
10^3
10^4
10^5
10^6
10^7
0
20
40
60
80
Amount of p3-HEL fusion protein (%)

Figure 3



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/00030

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/70, C12N 15/11, C12N 15/62, C12N 7/01
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12Q, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

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MEDLINE, BIOSIS, DBA SCISEARCH, WPI, EPODOC, PAJ, PCI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEMS Microbiology Letters, Volume 125, 1995, Marta Duenas et al, "Novel helper phage design: intergenic region affects the assembly of bacteriophages and the size of antibody libraries" page 317 - page 322 --	1-12
D,A	WO 9516027 A1 (BIOINVENT INTERNATIONAL AB), 15 June 1995 (15.06.95) --	1-12
D,A	EP 0614989 A1 (MORPHOSYS GESELLSCHAFT FÜR PROTEINOPTIMIERUNG MBH), 14 Sept 1994 (14.09.94) -- --------	1-12

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